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TRIPHOSPHOINOSITIDE BREAKDOWN AND DENSE BODY RELEASE AS THE EARLIEST EVENTS IN THROMBIN-INDUCED ACTIVATION OF HUMAN PLATELETS

F. RENDU*, P. MARCHE**, J. MACLOUF*, A. GIRARD** and S. LEVY-TOLEDANO*

*INSERM U 150, Hôpital Lariboisière, 6 rue Guy Patin, F-75010 PARIS
**INSERM U7, Hôpital Necker, 161 rue de Sèvres, F-75015 PARIS, France

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The activation by thrombin of human platelets prelabelled with ^{32}P induced a 30-40 % decrease in ^{32}P-triphosphoinositides (TPI) in the first 10 sec ; the decrease in the other ^{32}P-labelled phosphoinositides occured by 20-30 sec. At 10 sec., the intensity of these effects was maximum with 0.2-0.4 U/ml thrombin. Under these conditions, 53, 20 and 15 % of the dense granule, alpha-granule and lysosome constituants, respectively were released and thromboxane B_2 synthesis reached only 10 % of its maximum. Together with experiments carried out with chlorpromazine - or PGE_1 - treated platelets, our results suggest the existence of a close relationship between TPI-breakdown and dense body release which appear to be the earliest events resulting from the activation of human platelets by thrombin.

The physiological responses which follow the activation of platelets (i.e. cell shape change, aggregation, secretion) are thought to occur through the mobilization of intracellular Ca^{2+} (1-3). Since inositol phospholipids - phosphatidylinositol (PI) and its phosphorylated derivatives, the diphosphoinositides (DPI) and triphosphoinositides (TPI) - appear to be closely involved in processes of transmembrane Ca^{2+} fluxes and/or Ca^{2+} mobilization (4, 5), particular attention has been focused on the role played by these lipids during platelet activation.Changes in phosphoinositide metabolism, as measured by variations in the cell content of compounds involved in the phosphoinositide cycle and by variations in radioactivities associated with these compounds (in the case of prelabelled platelets), have been reported to occur within the first seconds that follow the stimulus-induced platelet activation (for review see 6). Moreover in human platelets, recent studies indicated that upon thrombin stimulation, polyphosphoinositides (TPI and DPI) might participate in the process of stimulus-activation coupling by initiating the phosphoinositide cycle (7-9). However, the exact role of the "phosphoinositide effect" in the throm-

ABBREVIATIONS : PI, phosphatidylinositol ; DPI ; phosphatidylinositol 4-phosphate or diphosphoinositide ; TPI, phosphatidylinositol 4,5-bisphosphate or triphosphoinositide ; PA, phosphatidic acid ; β-TG, β-thromboglobulin ; NAG, N-acetyl D-glucosaminidase.

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bin-triggered activation is still unclear as the previous data were either obtained with high concentrations of thrombin or given with little indication of the platelet reactivity. This prompted us to investigate the effect of lower thrombin concentrations on the phosphoinositide metabolism in human platelets in relation with platelet activation. For this purpose, after labelling of platelets with ^{14}C-serotonin and ^{32}P-orthophosphate, and activation by thrombin under various conditions, the measurements of aggregation, of the release of dense body, alpha granule and lysosome constituants and of thromboxane B_2 production were made in parallel with the measurements of radioactivities associated with the phosphoinositides.

MATERIAL AND METHODS

Blood was anticoagulated in 1:10 vol of ACD-C (10) and platelet rich plasma (PRP) was obtained by 10 min of centrifugation (100 g, 20°C). The PRP was divided in two parts : one part was incubated with 0.6 µM ^{14}C-serotonin (48 mCi/mmol - CEA Saclay, France), and the other part with 1:30 vol of carrier-free ^{32}P-orthophosphate neutralized in NaOH just before used (1 mCi/ml - Amersham, U.K.). After 90 min of incubation at 37°C, each of the PRP samples was layered onto a metrizamide gradient and centrifuged for 15 min at 1000 g (11). The metrizamide gradient platelets (MGPs) thus obtained, were washed by repeating the procedure on a similar metrizamide gradient. Each final MGP suspension was adjusted to 5.10^8 cells/ml in : NaCl, 140 mM ; KCl, 3 mM ; $NaHCO_3$, 12 mM ; glucose, 10 mM ; $MgCl_2$, 0.5 mM ; pH 7.4 ; 300 mOsm.

Aggregation and release on the one hand, and phosphoinositide metabolism as well as thromboxane B_2 synthesis on the other hand, were studied on the ^{14}C-serotonin- and ^{32}P-labelled MGP samples, respectively. Aliquots of 0.5 ml MGP were transferred to the aggregometer cuvettes and preincubated for 2 min at 37°C. Following thrombin addition and incubation for the designated times, the ^{14}C-serotonin and ^{32}P-labelled samples were transferred to tubes containing 0.1 ml ice cold EDTA 0.1 M and 2 ml ice cold chloroform/methanol/conc. HCl/EDTA 0.1 M (20/40/1/2, v/v/v/v), respectively for terminating reactions.

The ^{14}C-serotonin-labelled samples were immediately centrifuged for 30 sec in an Eppendorf centrifuge and the supernatant analyzed for ^{14}C-serotonin, beta-thromboglobulin (β-TG) (11), N-acetyl-glucosaminidase (NAG) (12).

Aliquots (0.2 ml) of the ^{32}P-labelled samples thus diluted in the organic mixture were used for thromboxane B_2 determinations which were performed by radioimmunoassay using ^{125}-I-labelled derivative according to Maclouf et al (13). The rest of ^{32}P-labelled samples were processed for phospholipid extraction and separation as previously described (14). Such a one dimensional thin-layer chromatography allowed the measurements of ^{32}P incorporated into TPI, DPI and PI. Since in this chromatography, phosphatidic acid (PA) has been shown to migrate with the solvent front (14), scraping of the corresponding area allowed us also to estimate the ^{32}P associated with PA (see below).

At least 3 control samples (i.e. without addition of thrombin) were measured periodically during the experiments which never lasted more than two hours ; they did not differ from each other by more than 8 % and 3 % for ^{32}P- and ^{14}C-serotonin-labelled samples, respectively. In another type of controls, ^{14}C-serotonin-labelled samples were transferred to tubes containing 0.1 ml ice cold EDTA 0.1 M and thrombin, and were immediately centrifuged. These controls did not differ from the preceding ones by more than 2 %, for all granule markers. This indicates that with respect to the parameters measured, i) the platelet function did not vary significan-

tly during the experiments and ii) reactions were efficiently stopped at the designated times.

RESULTS AND DISCUSSION

Using the labelling procedure described above, the ^{32}P-radioactivity associated with TPI, DPI, PI and compounds which migrated with the solvent front, represented 32 ± 4, 31 ± 2, 27 ± 3 and 10 ± 1 % (mean ± SEM, n = 6), respectively of the total phospholipid radioactivity in control unstimulated platelets. A similar distribution in ^{32}P-phosphoinositides has already been reported (15).

The time-course of variations in ^{32}P associated with the phosphoinositides during the platelet stimulation by thrombin (0.2 U/ml) is shown in Figure 1A. 10 sec after thrombin addition, ^{32}P-TPI decreased significantly by 30 %. Then ^{32}P-TPI increased and reached 120 and 140 % of the control value after 30 and 120 sec, respectively. The variations in ^{32}P-DPI and ^{32}P-PI were very similar : after a 20-30 % decrease by 20-30 sec, ^{32}P-DPI and ^{32}P-PI increased and had almost recovered control values at 120 sec. Figure 1A also shows a rapid and marked increased in the radioactivity associated with compounds which migrated with the solvent front. This was observed as soon as 10 sec after thrombin addition. A very rapid increase in ^{32}P-PA during platelet activation has been well documented (see ref. 6 for review). Therefore, it is likely that ^{32}P variations we measured at the

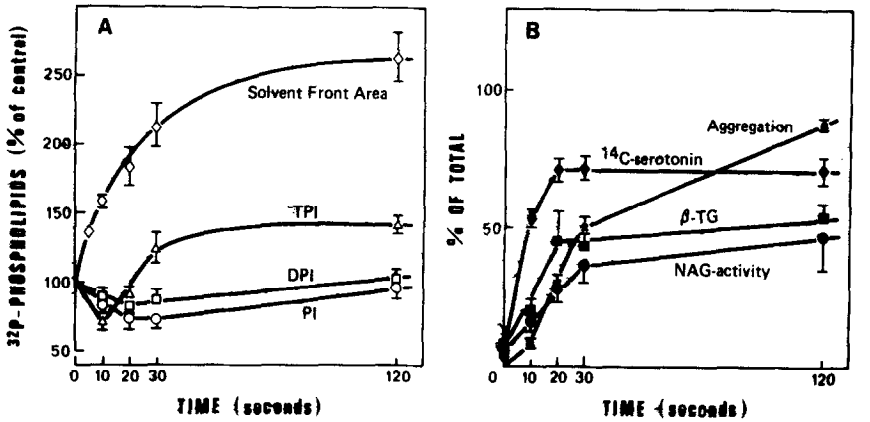

Figure 1 Time-dependent changes induced by thrombin on ^{32}P-phospholipids, aggregation and release reaction. ^{32}P- and ^{14}C-serotonin-prelabelled human platelets (5.10^8 cells/ ml) were incubated with thrombin (0.2 U/ml) for designated times. A : Variations of ^{32}P-associated with phospholipids. Results are expressed as the percent changes from zero time ; values are the mean + SEM of duplicate determinations for each of 4-5 platelet preparations. B : Aggregation (×) and release of ^{14}C-serotonin (◆), β-thromboglobulin (■) and N-acetyl- glucosaminidase (●). Results are expressed as per cent of light transmission for aggregation and as per cent release of total platelet content for each granule constituant. Values are the mean ± SEM of 6-8 platelet preparations.

solvent front represented mainly ^{32}P-PA variations. Such variations of radioactivities associated with PI and/or polyphosphoinositides have been already described in human and equine platelets stimulated by thrombin under different experimental conditions (8, 9, 16-18).

The parallel time-course for "typical" platelet responses is represented in Figure 1B. The platelet release was extremely rapid and was nearly completed after 30 sec, as was the case for the variations in ^{32}P-phosphoinositides. 10 sec after the addition of thrombin (0.2 U/ml), when the intensity of aggregation was only 9 %, the release of ^{14}C-serotonin was already 80 % of its maximum. By contrast, the liberation of other granule constituants (i.e. β-TG from alpha granules and NAG activity from lysosomes) were only 37 % of their maximum value. These data are in agreement with those obtained by Akkerman et al. (19) who used gel-filtered platelets. The important release from dense bodies as compared to that from other granules was ascertained by the measurement (with the luciferin/luciferase system) at 10 sec of the release of adenine nucleotides (ATP + ADP) which represented 80 % of the maximum releasable value (results not shown). Thromboxane B_2 levels were below 1 ng/ml in unstimulated platelets and reached 15-20, 50-100 and 200-250 ng/ml at 10, 30 and 120 sec, respectively following thrombin addition (0.2 U/ml).

The platelet responses were also measured at 10 sec as a function of the thrombin concentration. A significant loss of ^{32}P from TPI could be observed with 0.1 U/ml thrombin, but the maximum decrease in each ^{32}P- phosphoinositide was reached with 0.2-0.4 U/ml thrombin (Figure 2A), concentrations at which the aggregation and the release reaction reached their maxi-

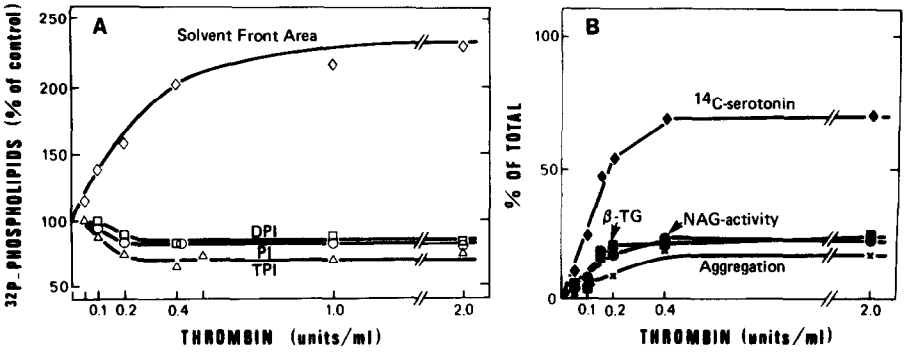

Figure 2 The effect of various thrombin concentrations on ^{32}P-phospholipids, aggregation and release reaction. ^{32}P- and ^{14}C-serotonin-prelabelled human platelets (5.10^8 cells/ ml) were incubated for 10 sec at 37°C with different concentrations of thrombin (0.05-2 U/ml). Symbols are as in Figure 1 ; values are the mean of duplicate determinations performed on 3 different platelet preparations. A : Variations of ^{32}P associated with phospholipids ; results are expressed as per cent changes versus control incubated without addition of thrombin. B : Aggregation and release ; results are expressed as in Figure 1.

mum (Figure 2B). Such a thrombin-dependence closely resembles that reported for variations in ^{3}H-arachidonic acid labelled-PI and -diacylglycerol as well as in the release of serotonin (16, 20). The significant difference in the amounts of granule constituants liberated at 10 sec (50 % from dense bodies and 25 % from both alpha-granules and lysosomes) confirms previous observations that dense bodies appear much earlier than other granule populations during platelet activation by thrombin (7, 19). It is noteworthy that in the absence of thrombin, only 4, 8 and 4 % of ^{14}C-serotonin, β-TG and NAG activity were released, respectively. This indicated that the present experimental conditions permitted the obtention of well preserved platelets as confirmed by their responsiveness to low thrombin concentrations.

The effects of inhibitors which affect Ca^{2+}- and/or cyclic AMP-dependent responses were also investigated 10 sec after thrombin stimulation (Table 1). Treatment of platelets with chlorpromazine (CPZ) prior to thrombin addition did not prevent the loss of ^{32}P from all three phosphoinositides. This agrees with what has been reported in trifluoroperazine-treated horse platelets (21) and suggests that the thrombin-induced polyphosphoinositide breakdown is not a consequence of the internal Ca^{2+} mobilization. CPZ completely abolished the aggregation and inhibited strongly the release of β-TG but only slightly that of ^{14}C-serotonin. By

TABLE 1. The effects of chlorpromazine (CPZ) and PGE_1 pretreatment on thrombin-induced changes in platelet ^{32}P-phospholipids, aggregation and release

	Thrombin	Thrombin + CPZ	Thrombin + PGE_1
Δ% vs	Saline	CPZ	PGE_1
^{32}P-TPI	- 37	- 44	0
^{32}P-DPI	- 26	- 37	+ 18
^{32}P-PI	- 21	- 22	+ 20
^{32}P at the front	+ 66	+ 86	+ 68
Aggregation %	9	0	2
Release (% of total)			
^{14}C-5HT	53	43	17
β-TG	20	4	12

^{32}P-labelled platelets (5.10^{8} cells/ml) were prewarmed for one minute at 37°C in the aggregometer cuvette. Preincubation was prolonged for one minute with saline, CPZ (100 μM) or PGE_1 (0.24 μM) before the addition of thrombin (0.2 U/ml). Reaction was stopped 10 sec later. Results are expressed as percent decrease (-) or increase (+) versus controls (saline- or drug-treated ^{32}P-platelets, without addition of thrombin). Mean of 2 experiments.

contrast under our experimental conditions, pretreatment of platelets with PGE_1 did prevent the phosphoinositide breakdown elicited by thrombin and even enhanced the ^{32}P-labelling of DPI and of PI. Dibutyryl cyclic AMP and/or prostacyclin have been described to inhibit the breakdown of PI and (although more slightly) that of TPI as well as the generation of diacylglycerol (16, 21-23). Thus it is likely that the effects of PGE_1 could be ascribed, at least in part, to its ability to increase the intracellular level of cyclic AMP (24, 25). In our experiments, PGE_1 strongly reduced the aggregation and the release of ^{14}C-serotonin but was less effective on the release of alpha-granule constituants. It is of interest that the compound which inhibited the thrombin-induced ^{32}P-TPI breakdown also inhibited the release of ^{14}C-serotonin whereas the compound which did not affect ^{32}P-TPI had little effect on the release of ^{14}C-serotonin.

Neither chlorpromazine nor PGE_1 signigicantly affected the ^{32}P-labelling that we measured at the solvent front (Table 1), thus suggesting that ^{32}P-PA can increase even in the absence of phosphoinositide degradation. A discrimination between PA formation on the one hand and PI breakdown, aggregation and dense granule secretion on the other hand, has been already described (26).

The present results provide evidence that in human platelets, low concentrations of thrombin cause a rapid degradation of ^{32}P-phosphoinositides and that the earliest event appears to be ^{32}P-TPI breakdown. Several mechanisms (discussed in ref 18) can account for ^{32}P-TPI breakdown. However, the possibility of its phosphodiesterasic cleavage seems to be most likely since increases in the products of this enzymic reaction (i.e. diacylglycerol and ^{32}P-labelled inositol triphosphate) have been separately reported to occur within the first 10 sec following thrombin stimulation (9, 16). Although we could detect a significant thromboxane B_2 formation at the early times of platelet activation, this represented, at 10 sec, only 10 % of the maximum synthesis, in contrast with the dense body liberation which reached 80 % of the maximum releasable value. Our observation that TPI breakdown was accompanied by the liberation of dense granule constituants strongly suggests the existence of a close relationship between the two phenomena. This lends support to the hypothesis that the agonist-induced breakdown of TPI can be considered as an early event occuring in Ca^{2+} mobilization (18, 27, 28). However, with regard to the thrombin-induced activation of human platelets, it still remains to establish whether TPI breakdown precedes the release of dense bodies.

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